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13. ABSTRACT (Maximum 200 Words)

The endothelial receptor tyrosine kinase Tie2 is highly overexpressed in breast cancer and its overexpression is significantly correlated with metastasis and poor outcome. Vascular endothelial growth inhibitor (VEGI), a novel antiangiogenic agent that inhibits breast cancer growth and angiogenesis, can maintain G_0/G_1 arrest of synchronized endothelial cells (EC) and induce apoptosis in proliferating EC. We have observed that VEGI can regulate expression of Tie2, reducing protein level about 50% during growth arrest, but rapidly eliminating its expression in proliferating cells induced by VEGI to undergo apoptosis. This proposal examines the hypothesis that VEGI inhibits angiogenesis by inhibition of Tie2 signaling. We have shown that VEGI signaling is dominant to AKT signaling, as VEGI can attenuate Angiopoietin-1(Ang1)-mediated activation of Akt in apoptotic cells, and that prior stimulation of Tie2 by Ang1 cannot prevent VEGI-mediated downregulation of Tie2 or prevent apoptosis. Further, overexpression of Tie2 only slightly attenuates VEGI-mediated apoptosis, while overexpression of myr-AKT does confer some protection. Mechanistically, we have determined that the downregulation of Tie2 is controlled by both decreased RNA expression as well as group I caspase-mediated cleavage of existing Tie2 protein during apoptosis. These data suggest that VEGI may be an effective agent against Tie2 overexpressing tumor vasculature.

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INTRODUCTION

The endothelial receptor tyrosine kinase Tie2 plays an important role in angiogenesis[1] and endothelial survival[2,3]. Tie2 is overexpressed in breast cancer vessels[4,5], where it is correlated with poor outcome[6]; further, therapies targeting Tie2 have shown promise in animal models[7-9], suggesting that downregulation of Tie2 may be therapeutically beneficial. Vascular Endothelial Growth Inhibitor (VEGI) is a novel endogenous anti-angiogenic agent that has been shown to inhibit breast cancer growth and angiogenesis[10,11]. This inhibition of angiogenesis may be explained my our *in vitro* observation that VEGI can induce or maintain a G_0/G_1 growth arrest in endothelial cells that are in G_1, but induce apoptosis in proliferating endothelial cells[12]. The current grant is based on our initial observation that VEGI can moderately decrease Tie2 levels in arrested cells, but nearly eliminate Tie2 expression during apoptosis, and investigates the hypothesis that VEGI exerts its inhibitory effects on angiogenesis by modulation of Tie2 signaling.

BODY

Progress for tasks outlined in Statement of Work: Most of the following data is included in the manuscript we are preparing for submission, which is attached; please refer to it for detailed methods and discussion of results.

Task 1: Determine whether downregulation of Tie2 is functionally significant during apoptosis and angiogenesis (Months 1-15)

A. Develop Tie2 plasmids – Despite initial difficulties, we have obtained a functional expression vector for Tie2 and created probes.

B &C. Evaluate the ability of ectopic Tie2 or AKT to prevent apoptosis – Although the increased detection of both basal and Ang1-stimulated phosphorylated AKT suggests that the ectopic protein is active, overexpression of Tie2 does not effectively prevent VEGI-mediated apoptosis, as measured by caspase cleavage (decreased only about 10% in Tie2 overexpressing cells) (see Fig. 1A); similar results were reported last year with caspase activity assay. We now think that this may be due to VEGI's inhibition of both basal and Ang1-stimulated AKT signaling in the apoptotic population, as shown in Task 1D (Fig. 2A). We reported last year that overexpression of myristoylated Akt (myr-Akt) was also not very effective in overriding VEGI-mediated apoptosis, but were concerned about transfection efficiency, which is a maximum of 50% with these cells. As a result, I co-transfected with a Ds-Red vector and sorted on the basis on Ds-Red expression to enrich for the transfected population, then treated the Ds-Red positive cells with VEGI; AKT appears to partially prevent

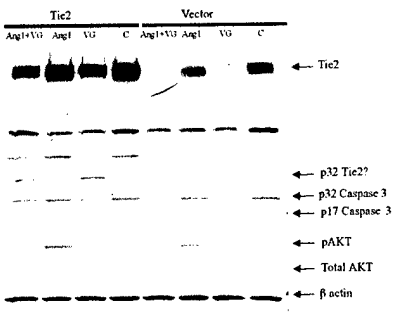

Figure 1. Overexpression of Tie2 does not attenuate apoptosis. Lysates from ABAE transfected with pCMV-Tie2 or vector were subjected to Western analysis as indicated.

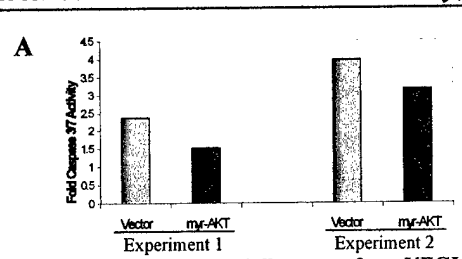

Figure 2. Myr-Akt can partially rescue from VEGI-mediated apoptosis. A, Twenty-four hours post-transfection, ABAE co-transfected with pcDNA3-myr-Akt and pCMV-Ds-Red were sorted by flow cytometry and treated with VEGI or control buffer for 12 hours, and caspase 3/7 activity measured by Z-DEVD-Rho110 cleavage. B, Cells transfected with myr-Akt and pCMV-Tie2 were analyzed by Western for pAKT and total AKT.

VEGI-mediated apoptosis (see Fig. 2A, up to 30 or 40%, compared to the 20% inhibition observed in unsorted populations (data presented Year 1). Although myr-AKT phosphorylation is high in untreated and VEGI-arrested cells, VEGI may retain the ability to cleave and/or possibly dephosphorylate the myrAKT in the apoptotic, detached population (Fig. 2B).

D. Evaluate the effect of Ang1 on Tie2 Expression and apoptosis – We next determined which signaling pathway, VEGI or Ang1, is dominant. We found that i) Ang1 cannot prevent VEGI-mediated Tie2 downregulation (reported last year; see manuscript), ii) Ang1 does not effectively protect against VEGI-mediated apoptosis (reported last year; see manuscript), and, most importantly, this seems to be due to the fact that iii) VEGI decreases basal but not Ang1-stimulated phosphorylation of Akt in VEGI-arrested attached cells, while it can prevent both basal and Ang1-stimulated Akt phosphorylation in the detached apoptotic population, even while some Tie2 is present (Fig. 3A). Interestingly, in separate work, my former mentor and current collaborator LuYuan Li has performed microarray analysis on endothelial cells treated 5 hours with VEGI, and discovered that PTEN, the phosphatase that downregulates the PI3K/Akt pathway, is upregulated 9.6-fold during VEGI-mediated apoptosis, but only 4.5-fold during VEGI-mediate growth arrest (personal communication). I have confirmed this upregulation of PTEN protein by VEGI (Fig. 3B; though the fig is faint, PTEN is detectable by 4h and continues to increase through 12h VEGI), and obtained PTEN overexpression (gift of Dr. Kenneth Yamada) and siRNA constructs (gift of Dr. Qi Wan) which I have grown up and isolated to evaluate the possible role of PTEN in mediating VEGI's effects. This is of particular importance, as we have also found that endothelial cells treated with the PI3K inhibitor LY294002 ('LY'), which prevents Akt phosphorylation and subsequently induces apoptosis, can also lead to downregulation of Tie2 in a fashion similar to that of VEGI, i.e. decrease of full-length as well as appearance of the cleavage product (heavy arrow) (Fig. 3C – see Task 2B below for description of cleavage product.) It appears then, that VEGI not only prevents Tie2-mediated Akt signaling (possibly by overexpression of PTEN), but that maintenance of this PI3K/Akt pathway may be required to sustain expression of Tie2. While these experiments represent a deviation from the original SOW, it is of particular importance to determine whether inhibition of this pathway by other means, i.e. overexpression of PTEN or dominant negative Akt (gift of Dr. Bob Glazer), also leads to loss of Tie2, suggesting that mechanistically, VEGI induces apoptosis via inhibition of the Akt pathway which then leads to subsequent downregulation of Tie2. Obtaining these data, then, may play a critical role in full elucidation of the regulation of Tie2 by VEGI, which is the focus of this grant.

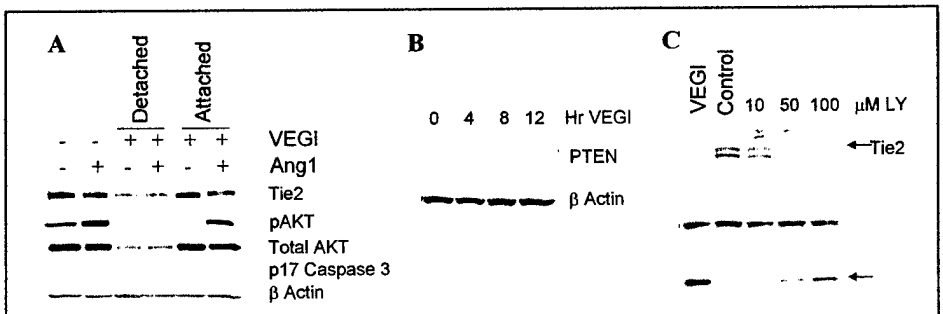

Figure 3. Lysates from ABAE treated with VEGI (A, B) or PI3K-inhibitor LY294002 (C) were subjected to Western analysis of indicated proteins.

Task 1, Part E: Evaluate the ability of VEGI to inhibit Ang1-mediated *in vitro* tubule formation – While we had initially planned these experiments for the second year, we have been focusing on the apoptosis effects and the experiments (years 2 and 3) proposed for Task 2. Following submission of the attached manuscript, I will return to this experiment in year 3.

Task 2: Determine whether Tie2 expression is controlled at the RNA or protein level (Months 16-28) – Due to the initial difficulties in obtaining a functional Tie2 vector, we began to evaluate

control of Tie2 protein levels in the first year. These data have turned out to be more complex (and intriguing) than initially thought, so considerably more time than originally allotted has been spent on this task.

B. Evaluate protein stability under conditions of growth arrest and apoptosis – We compared the downregulation of Tie2 in response to VEGI with the protein synthesis inhibition by cycloheximide (CHX) and observed that Tie2 loss is more rapid following VEGI than with treatment by CHX, suggesting that Tie2 is less stable in the presence of VEGI. In addition, we observed the appearance of an approximately 32kDa product detected by our C-terminal antibody (Santa Cruz) in VEGI-treated apoptotic cells, but not in untreated or VEGI-arrested cells, further suggesting an active mode of degradation during apoptosis. These data were reported in the Year 1 progress report (also see manuscript). Additional data that further support that this band represents a cleavage product of Tie2 are i) it can be detected by second Tie2 C-terminal antibody (GenEx, not shown); ii) its appearance cannot be prevented, but is further augmented, by inhibition of protein synthesis by CHX (see Year 1 report); iii) its appearance is concomitant with loss of full-length Tie2 (Figs. 1A and 3C); and iv) its detection is increased in Tie2-overexpressing cells undergoing VEGI-mediated apoptosis (Fig. 1A). Surprisingly, however, when I subcloned the Tie2 cytoplasmic region and overexpressed in cells, the truncated version did not appear to be cleaved in response to VEGI; we postulate that this may be due to the inability of the cytoplasmic fragment to be targeted to the membrane and processed similarly to p140 Tie2 (not shown). Unfortunately, despite numerous attempts, I have been unable to immunoprecipitate sufficient quantities of the 32kDa protein to perform sequencing to a) confirm it is a Tie2 cleavage product, and b) determine the N-terminal residue to identify cleavage site, but these efforts are continuing.

We next sought to determine the mechanism by which Tie2 cleavage occurs. None of the proteasome (MG132, lactacystin; data shown in Year 1 report), calpain (LL-CHO, ALLN) or metalloprotease (BB-94) inhibitors tested prevented loss of full length Tie2 or prevented appearance of the cleavage product (Fig. 4A, B). I also addressed whether lysosomal degradation might be involved; I found that, first, the lysosomal poison chloroquine was too toxic to the cells and instead tested whether lysosomal cathepsin inhibitors could prevent loss of Tie2. The cathepsin B inhibitor Ca074-Me but not the cathepsin L inhibitor Napsul-Ile-Trp-CHO slightly attenuated loss of p140 Tie2, as well as very slightly decreased the appearance of the 32kDa product (Fig. 4C) at low doses but not higher doses; the capthespin/calpain inhibitors E64 and ALLM gave similar results (not shown). This slight attenuation of VEGI's effects on Tie2 was also accompanied by slight attenuation of PARP cleavage (not shown), suggesting that apoptosis was slightly decreased (though at higher doses it slightly synergized with VEGI). It therefore appears that activation of cathepsin B may precede the activation of caspases during VEGI-mediated apoptosis, but the significance of this remains unclear.

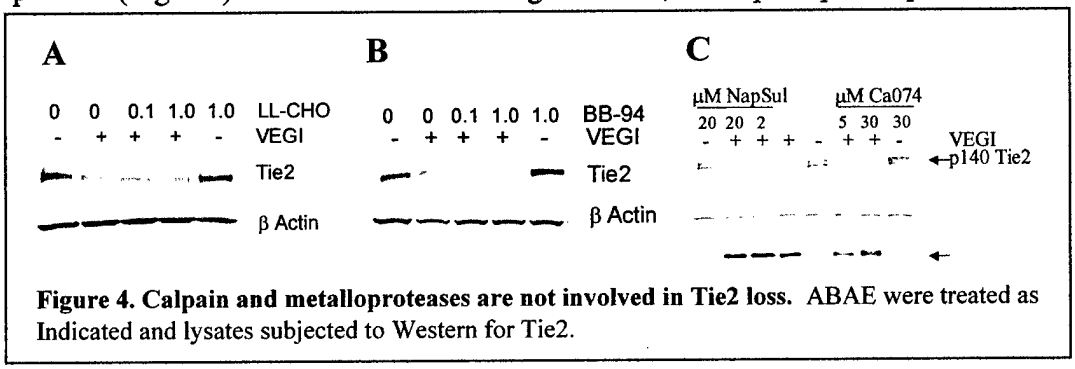

Figure 4. Calpain and metalloproteases are not involved in Tie2 loss. ABAE were treated as Indicated and lysates subjected to Western for Tie2.

We also addressed whether caspases might be involved in the loss of Tie2. We reported last year that the broad spectrum caspase inhibitor ZVAD did not appear to significantly prevent loss of full-length Tie2; however, during our study of the 32kDa cleavage product, it became

evident that while ZVAD only partially restores detection of p140 Tie2, it completely prevents the appearance of the 32kDa cleavage product (Fig. 5A). It was later determined that there are at least two mechanisms by which VEGI causes downregulation of Tie2, cleavage of existing Tie2 and decreased synthesis of new Tie2 due to lower mRNA (see Task 2a below). Examination of the Tie2 cytoplasmic region identified three potential caspase 3 or 7 motifs (DXXD, GELE[13]), one caspase-8 LETD motif, and one LXXD motif similar to the sequence preferred by group I caspases (which include caspases 1, 4, 5, 11, 12, 13, 14). We then tested a variety of cell-permeable specific caspase inhibitors for their ability to protect p140 Tie2 and prevent the appearance of the 32kDa product, and found that the group I caspase inhibitor YVAD as well as the caspase 4 inhibitor LEVD were effective, although not as significantly as ZVAD, whereas caspase 3/7, 6, and 8 inhibitors had no effect (Fig 5A&B). ZVAD and LEVD also eliminated the detection of the 32kDa band in response to LY294002 (not shown). To further confirm that Tie2 may be a target for caspases, I next performed *in vitro* caspase assays using a 42kDa recombinant protein corresponding to the cytoplasmic region of human Tie2 as substrate ('p42 rhTie2'). As seen in Fig. 5C (and manuscript), caspase 4, and to a much lesser extent, caspases 1 and 5, cleaved Tie2 to give rise to a product nearly identical in size to the 32kDa band produced *in vivo* in response to VEGI and which corresponds to the predicted molecular weight (31.2kDa) of a protein cleaved at the LXXD site of Tie2. These data suggest that Tie2 may be a target for caspase 4 or other group-1 caspases during VEGI-mediated apoptosis.

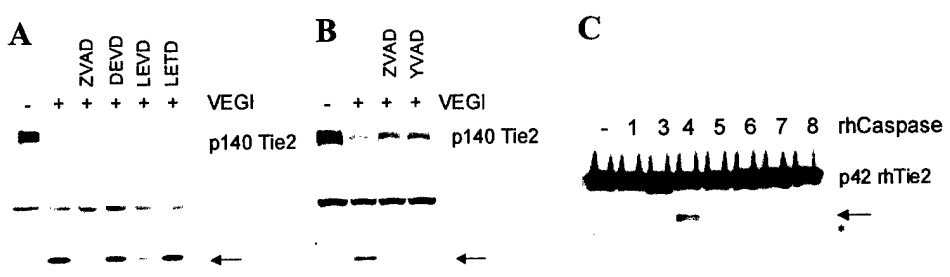

Figure 5. Group I caspase mediates Tie2 cleavage. A & B, ABAE cells were preincubated for 1 hour with the indicated caspase inhibitor, then treated with VEGI overnight and lysates analyzed for Tie2. C, Recombinant p42 Tie2 cytoplasmic region was subjected to in vitro cleavage using the indicated recombinant caspase and analyzed by Western. Arrows indicate p32 cleavage product. Askterisk denotes smaller product cleaved by caspase 7 thought to be artifact.

Task 2, A: Evaluate RNA levels, stability, and transcription (Months 16-28)

While we had initially intended to perform these experiments by RNAse protection, we have switched to RT-PCR due to a) initial difficulties in obtaining a usable Tie2 plasmid, and 2) RT-PCR requires far less RNA, and therefore fewer cells, for analysis, which is of importance since the recombinant VEGI protein is in limiting quantities. Semi-quantitative RT-PCR on RNAs isolated from proliferating or confluent cells treated for 6 hours with VEGI to induce apoptosis or arrest, respectively, shows that, similar to the Tie2 protein increases with increasing confluence observed by us and others (see manuscript), Tie2 mRNA levels are higher in confluent cells than in proliferating cells (Fig. 6). We found that VEGI causes a decrease in Tie2 mRNA in cells undergoing arrest, which are slightly further decreased in cells undergoing apoptosis. By contrast, cells induced to undergo apoptosis using 2-methoxyestradiol (2-ME) do not exhibit decreased Tie2 mRNA (or protein). Similar results were obtained using a second set of primers in a semi-

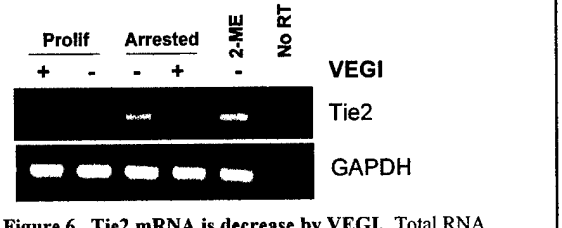

Figure 6. Tie2 mRNA is decrease by VEGI. Total RNA isolated from proliferating or arrested ABAE cells treated 5 hours with VEGI was subjected to semi-quantitative RT-PCR.

quantitative RT-PCR, although the limitations of this method are noted. I am currently working out conditions for a real-time PCR assay using SYBR green on our core facility's cycler to better quantify changes at the RNA level. In addition, I have isolated RNAs from LY294002-treated ABAE to determine whether Tie2 mRNA is similarly regulated, which will be quantified by this assay. The next issue to address is the mechanism by which the RNA is decreased. In order to determine whether stability is altered, I have treated cells with the RNA synthesis inhibitor actinomycin D, VEGI, or a combination of the two agents, and isolated the RNAs; this experiment has been performed twice, and the RNAs will be subjected to the real-time RT-PCR once the assay has been validated.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that Ang1 cannot override downregulation of Tie2 by VEGI or prevent VEGI-mediated apoptosis, suggesting that VEGI signaling is dominant
- Accordingly, determined that VEGI prevents both basal and Ang1-stimulated phosphorylation of Akt in apoptotic cells, and therefore overexpression of Tie2 cannot rescue cells; this may be due to upregulation of PTEN
- Overexpression of myr-AKT can partially rescue cells from VEGI-mediated apoptosis
- Made the novel observation that basal Tie2 expression may require an intact PI3K pathway
- Determined that VEGI regulates Tie2 at two levels: decreased synthesis due to decreased RNA levels during both growth arrest and apoptosis, and group I caspase cleavage of existing Tie2 protein during apoptosis

REPORTABLE OUTCOMES

1. **Metheny-Barlow, L.J.**, Laverriere, E.K., and Li, L.Y.: Regulation of Tie2 expression and function by Vascular Endothelial Growth Inhibitor (VEGI). Minisymposium presentation at the 95th Annual Meeting of the American Association for Cancer Research (March, 2004) and received an AACR-Takeda Scholar-In-Training Award; Proc. Am. Asso. Cancer Res. 45:1107, 2004.
2. **Metheny-Barlow, L.J.** and Li, L.Y.: The enigmatic role of angiopoietin-1 in tumor angiogenesis. Cell Res., 13(5):309-17, 2003. (Review article, see attached abstract)
3. **Metheny-Barlow, L.J.,** Laverriere, E.K., and Li, L.Y.: Regulation of Tie2 expression and function during VEGI-mediated apoptosis. In preparation, 2004; see attached manuscript.

CONCLUSIONS

In the present report, we have analyzed the regulation of the pro-angiogenic Tie2 receptor and its function by the anti-angiogenic molecule VEGI. The data show that VEGI completely prevents Tie2 signaling during apoptosis, suggesting that VEGI may be effective against Tie2-overexpressing tumor vasculature. Not surprisingly, overexpression of Tie2 can only very slightly attenuate VEGI-mediated apoptosis, while overexpression of myr-AKT does confer some protection. Mechanistically, we have determined that the downregulation of Tie2 is controlled by both decreased RNA expression as well as group I caspase-mediated cleavage of existing Tie2 protein during apoptosis. Further, we have data to suggest that a functional PI3K pathway may be required for sustained Tie2 expression; importantly, it has been previously shown that basal Tie2 expression is required for endothelial viability. We plan to further investigate this to determine whether targeting the PI3K/Akt pathway may be another strategy to eliminate Tie2 overexpression and signaling that is associated with tumor vasculature, and/or to enhance the effects of VEGI. Understanding of such interactions between positive and negative regulators of angiogenesis is critical to the refinement of VEGI and other anti-angiogenic agents for cancer therapies.

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Regulation of Tie2 expression and function by Vascular Endothelial Growth Inhibitor (VEGI)

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Subject codes:

ABSTRACT

The process by which a tumor converts from an avascular state to a vascular state is thought to occur by the ability of the tumor to locally alter the balance of pro-angiogenic factors and the inhibitor molecules that maintain the quiescence of the vasculature. The purpose of the current study is to analyze the relationship between the endothelial receptor tyrosine kinase Tie2 and the anti-angiogenic agent Vascular Endothelial Growth Inhibitor (VEGI). Tie2 is overexpressed on the vasculature of breast cancer and its overexpression has a significant correlation with metastasis and poor outcome. We have previously shown that, *in vitro*, antisense inhibition of Tie2 expression leads to endothelial apoptosis. Vascular Endothelial Growth Inhibitor (VEGI), a novel anti-angiogenic agent that inhibits breast cancer growth and angiogenesis, can maintain G_0/G_1 arrest of synchronized endothelial cells and induce apoptosis of proliferating endothelial cells. Here, we show that VEGI can regulate the expression of Tie2, reducing the protein level about 50% during VEGI-mediated growth arrest, but rapidly nearly eliminating its expression in proliferating cells induced by VEGI to undergo apoptosis. We therefore hypothesized that VEGI induces apoptosis by inhibition of Tie2 survival signaling. We determined that the apoptotic signaling by VEGI appears to be dominant, as VEGI can attenuate Angiopoietin-1(Ang1)-mediated phosphorylation of Akt, and prior stimulation of Tie2 by Ang1 cannot prevent VEGI-mediated downregulation of Tie2 or appreciably inhibit VEGI-mediated induction of apoptosis. The decrease in Tie2 levels during VEGI-mediated apoptosis is more rapid than that observed following inhibition of protein synthesis by cycloheximide, suggesting that Tie2 is degraded. This degradation appears to occur by a caspase 4-like caspase, since the broad-spectrum caspase inhibitor ZVAD as well as

group I caspase inhibitors YVAD and LEVD, but not DEVD or IETD, can attenuate this degradation, and caspase 4 can cleave Tie2 in an *in vitro* cleavage reaction. Analysis of Tie2 mRNA by RT-PCR suggests that Tie2 mRNA levels are reduced in response to VEGI. Our data demonstrate that VEGI-induced apoptotic signaling is dominant to Ang1-Tie2 survival signaling and suggest that VEGI may be an effective anti-angiogenic therapeutic agent for Tie2-overexpressing tumors.

Key words: angiogenesis, apoptosis, endothelial, Tie2, AKT

INTRODUCTION

Tie2/Tek is an endothelial-specific receptor tyrosine kinase that plays a crucial role in the development of the embryonic vasculature[1]. There are several ligands for Tie2, including: Angiopoietin-1 (Ang1), which is thought to be the main agonistic ligand for Tie2, and Angiopoietin-2 (Ang2), which at low concentrations appears to act as an antagonist to Ang1[2] but at higher concentrations can act as an agonist[3,4]. Various *in vitro* studies have demonstrated that Ang1 stimulation of Tie2 promotes endothelial cell migration[5], sprouting[6], and tubule formation[7] and stabilizes tubule networks[8]. In addition, stimulation of Tie2 by Ang1 has been shown to protect endothelial cells from a variety of apoptotic insults[8-10], at least partly through activation of the PI3K/Akt pathway[11-13]. Ang2, when present at high concentration, has also been shown to promote survival during serum deprivation-induced apoptosis by stimulation of Tie2 and subsequent activation of PI3K and Akt[3]. Importantly, Tie2 has also been shown to act as a basal survival factor for endothelial cells in a ligand-independent fashion, as elimination of Tie2 by antisense oligonucleotides[7] or RNAi[14] results in induction of apoptosis *in vitro*. In addition, conditional knockout of Tie2 *in vivo* results in endothelial cell apoptosis[15], further suggesting that Tie2 is necessary for endothelial survival.

Tie2 is expressed at low levels and is phosphorylated in normal quiescent endothelium [16], where it is postulated to maintain vessel stability. Interestingly, however, Tie2 has been shown to be upregulated on the vasculature of many tumors ([17]ref, ref, ref), where it is correlated with poor prognosis[18]. The importance of Tie2 in tumor angiogenesis has been confirmed by protocols devised to inhibit Tie2 *in vivo*. Injection of purified recombinant soluble Tie2 resulted in growth inhibition and decreased vascularization of a mammary tumor

in a rat cutaneous window assay[19]. In addition, adenoviral delivery of soluble Tie2 decreased murine mammary adenocarcinoma growth and metastasis[20]. Retroviral dominant negative Tie2 also inhibited growth of murine mammary xenograft tumors[21]. Taken together, these results suggest that Tie2 is an attractive target for anti-angiogenic therapies for breast cancer.

Vascular Endothelial Growth Inhibitor (VEGI), also known as TL1, is a recently identified angiogenesis inhibitor isolated from a human umbilical vein endothelial cell (HUVEC) library[22,23]. VEGI is a 174 amino acid protein that shares 20-30% identity with TNF family members. Expression of this molecule is predominantly seen in a subset of endothelial cells such as HUVEC and human venous endothelial cells. *In vitro*, VEGI has been shown to inhibit the growth of HUVEC and adult bovine aortic endothelial (ABAE) cells but not vascular smooth muscle cells, cancer cells or NIH3T3 fibroblasts[23]. In addition, VEGI has been shown to inhibit the formation of capillary-like tubules of ABAE cells in collagen as well as prevent tubule formation induced by either VEGF or bFGF in the chick embryo chorioallantoic membrane assay[24]. Importantly, VEGI has shown promise as an anti-angiogenic therapeutic agent to prevent tumor growth when expressed in human breast and prostate tumor xenograft models[23,24]. The efficacy of VEGI has recently been demonstrated in a preclinical Lewis Lung Carcinoma model as well (LYL, in preparation). We have further determined that VEGI can elicit its inhibition of angiogenesis by exerting two distinct effects on endothelial cells: growth arrest of cells already in G_0/G_1, or induction of apoptosis in proliferating cells[25]. Taken together, these studies suggest that VEGI demonstrates promise as a broad-spectrum anti-angiogenic therapeutic agent.

We are currently investigating the mechanism of action of VEGI in the inhibition of angiogenesis. In the present study, we have analyzed the interplay of the pro-angiogenic

endothelial survival Ang1/Tie2 pathway and the induction of apoptosis by VEGI. We find that VEGI can rapidly downregulate Tie2 expression and that VEGI-induced apoptotic signaling is dominant to Ang1/Tie2 survival signaling. Further, we demonstrate that this loss of Tie2 is accomplished by both decreasing mRNA levels as well as degradation of existing Tie2.

METHODS

Chemicals – ALLN, LL-CHO, Lactacystin, rh active caspases 1, 3, 4, 5, 6, and 8, rbovine PARP, and the broad-spectrum caspase inhibitor ZVAD were from Biomol (Plymouth Meeting, PA). Cell-permeable caspase inhibitor peptides YVAD (caspase 1/group I caspases), IETD (caspase 8), LEVD (caspase 4), and DEVD (caspase 3/7) were from Biovision Labs (CA). rhAngiopoietin-1 and rhTie2p42 were from R&D Systems (Minneapoli, MN). LY294002, CHX and MG132 were from Sigma (St. Louis, MO).

Cell Culture – Adult bovine aortic endothelial cells (ABAE) were gifts from Peter Bohlen (Imclone, New York, NY) and were cultured in IMEM (Biofluids Biosource International, Rockville, MD), 10% FBS, and 1ng/ml basic fibroblast growth factor (BBRL). The cells were grown in humidified incubators, 5% CO_2, 95% percent air at 37° C. For all experiments, the cells were used between passages 9-16.

Antibodies – Antibodies to Tie2 (C-terminus), PARP, and x were from Santa Cruz Technologies (Santa Cruz, CA). N-terminal antibody to Tie2 was from Calbiochem (San Diego, CA). Antibody to β-Actin was from Chemicon International, Inc. (Temeculeh, CA).

Anti-caspase 3, AKT, p-AKT (S473), and p-AKT (Thr308) antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-6x-His was from R&D Systems.

Vectors – pcDNA3.1-myrAKT was a kind gift of Dr. Robert Glazer (Georgetown University). pCMV-SPORT-6-hTie2 was from Invitrogen (Carlsbad, CA). pCMV-Ds-Red was from Clontech (Palo Alto, CA). Transfection was performe with Effectene Reagent (QIAgen, xx).

Western blotting – Protein lysates were prepared using a RIPA buffer (recipe) with protease inhibitor cocktail (Roche). Protein concentration was measured by BCA reaction (Pierce, Rockford, IL) and equal amounts of protein analyzed on 8-16% or 12% Tris-Glycine gels and transferred to nitrocellulose PVDF Immobilon-P (Millipore, Billerica, MA) or Protran nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). Membranes were blocked in PBST-0.5% Tween containing 5% dry milk. Following incubation with primary antibody, blots were washed and incubated with HRP-conjugated donkey anti-rabbit or anti-mouse secondary antibodies (Amersham Bioscience, Piscataway, NJ). Blots were developed using Western Lightening Plus (Perkin Elmer) exposed to Blue Bio Film (Denville Scientific, Metuchen, NJ) or Hyperfilm ECL (Amersham Biosciences).

Apoptosis assays - Proliferating ABAE were plated into normal growth media at $5x10^3$ cells/well in 96 well plates and pretreated or not with Ang1 and the anti-6x-His crosslinking mAb for 1 hour, after which VEGI (90ng/nl) or control buffer was added to the media. After 16h, caspase 3/7 activity was measured using the ApoONE Homogeneous Caspase 3/7 Assay (Promega, Madison Wisconsin) by adding Z-DEVD-Rhodamine110 peptide in a lysis buffer

directly to the media. Fluorescence (EM 485nm, EX 550nm) was measured on a Wallac 1420 plate reader. Results presented are mean of triplicate wells from two experiments +/- SD. Alternatively, cells were plated in T-25 flasks and treated with VEGI or control buffer for 12 hours. Both floating and adherent cells were then collected, stained with AnnexinV-FITC (Oncogene) and propridium iodide and analyzed by flow cytometry in two independent experiments.

Angiopoietin-1 stimulation - Proliferating cells were treated with control buffer or with VEGI [90ng/ml] to induce apoptosis. After 7 hours, cells were stimulated for 20 minutes with Ang1 [200ng/ml] and Anti-6x-His cross-linking antibody [5ug/ml], and lysates prepared separately from attached and detached cells. Western analysis was performed for p-AKT and total AKT. For measurement of Tie2 levels, Ang1 protein was co-incubated with VEGI protein for 12 hours.

Protease inhibitors - . Inhibitors were tested in a dose response manner with doses ranging from 0.1-10.0 μM. After 12 hours lysates were harvested and subjected to Western blot analysis as indicated.

For caspase inhibitor assays, cells were preincubated for 1 hour with 8μM indicated peptide, then VEGI (90ng/ml) to culture and the cells incubated 16 hours. Lysates were isolated and analyzed by Western.

In vitro Caspase Assay – Recombinant human Tie2 cytoplasmic region (Upstate Cell Signaling Solutions, Inc.) and recombinant bovine PARP (Biomol) were used as substrates. Caspase

cleavages were performed *in vitro* using purified° active recombinant caspases purchased from Biomol as described previously. Briefly, 200ng of susbstrate was incubated with caspases (30°C) for 1 hour (PARP) or 6 hours (Tie2). Caspase reactions were carried out in CHAPs buffer (50mM HEPES pH7.4, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol, 10mM DTT). Cleavage reaction products were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Tie2 (Santa Cruz c-terminal Ab) or PARP.

RT-PCR - ABAE cells were treated as indicated and total RNA isolated using TRIzol (Invitrogen). 1μg RNA was reverse transcribed with M-MLV RT (Promega), and PCR-amplified using Tie2- or GAPDH-specific primers, and PCR products analyzed on 1.5% agarose gel. [Primer sequences.] Both primer sets span introns and do not amplify genomic DNA. Similar results were obtained using different Tie2 primer pairs. Cycling conditions used were, Tie2: 94°C 2', 1 cycle; 94°C 20", 56°C 20", 72°C 40", 35 cycles; 72°C 5' (ref); and GAPDH: 94°C 2', 1 cycle; 94°C 20", 56°C 20", 72°C 40", 30 cycles; 72°C 5' (ref).

RESULTS

Tie2 Protein Levels are Decreased During VEGI-Mediated Apoptosis

We analyzed Tie2 expression in endothelial cells treated with VEGI to undergo growth arrest or apoptosis. Using our previously established conditions[25], ABAE cells synchronized by density arrest and were replated at low density into VEGI-containing media to elicit the VEGI-arrested state. Analysis of lysates by Western demonstrates that, while increasing levels of Tie2 is detected in untreated samples, Tie2 levels remain low in the VEGI-arrested cells

(Figure 1A, left panels). Alternatively, when cells are released from density arrest and are then treated with VEGI when cells are actively proliferating, cells undergo apoptosis[25,26]; under these conditions we find that Tie2 levels are rapidly and drastically decreased compared to untreated proliferating cells (Figure 1A, middle panels). After about 48 hours, when the proliferating cell population (up to about 50% of total cells[25]) has undergone apoptosis, the growth curve levels off, and the remaining viable attached cells remain arrested[25]; when this population is analyzed for Tie2, we found that Tie2 levels were detectable, although at lower levels than that seen in untreated proliferating cells, similar to the decrease observed with VEGI-growth arrested samples. It has previously been reported that Tie2 levels increase with increasing confluence in vitro[27]; in order to determine whether the decreased expression of Tie2 we observed resulted from maintenance of low confluence due to growth arrest or whether Tie2 levels were actively downregulated, we treated ABAE cells that are already arrested by confluence with VEGI. Confluent density-arrested ABAE treated with VEGI for 24 hours show no discernable change in cell number[25], but also demonstrates a moderate decrease in Tie2 protein levels, suggesting that VEGI can regulate Tie2 independent of culture confluence (Figure1A, far right panel).

To confirm that Tie2 was eliminated specifically in the cells that undergo apoptosis in response to VEGI, proliferating ABAE were treated with VEGI or control buffer for 16 hours, after which cells the cells were harvested by either combining attached and detached cells ('All'), or isolating the detached, floating cells ('Detached') separately from the attached ('Adherent') cells from the same dish. Western analysis demonstrates that Tie2 is lost specifically from the detached apoptotic population (Figure 1B, third lane); we also observed the accumulation of a 32kDa species detected by a C-terminal antibody which we examined in

subsequent experiments. The detection of the active p17 subunit of caspase 3 specifically in this sample confirms that only the detached, not the adherent, population is apoptotic. Interestingly, Tie2 levels are moderately decreased in the adherent, arresting population compared to untreated controls, consistent with VEGI's ability to slightly attenuate Tie2 expression in growth arrested cells.

Ang1 cannot override the effects of VEGI

Treatment of endothelial cells with Ang1 has been shown to confer protection from a number of apoptotic insults[8-12]. In order to determine whether Ang1 or VEGI signaling was dominant, we next addressed whether pretreatment with Ang1 could reverse the effects of VEGI. We first asked whether Ang1 could reverse the downregulation of Tie2 by VEGI. Interestingly, we found that overnight exposure to Ang1 alone could slightly decrease detection of Tie2 (Figure 2A), which we attribute to degradation of the ligand-receptor complex following transduction of signal. Accordingly, we found that not only could Ang1 not prevent the loss of Tie2 in response to VEGI, but in fact led to a further decrease in the detection of Tie2, presumably due to the degradation suggested above.

We next addressed whether Ang1 could attenuate the induction of apoptosis by VEGI. For these experiments, proliferating ABAE were plated in a 96 well plate, treated for 1 hour with Ang1, then treated overnight with VEGI, after which apoptosis was measured using fluorescently labeled caspase 3/7 substrate. We found that even at levels of 1000ng/ml, Ang1 pretreatment could attenuate VEGI-induced apoptosis only by about 20% (Figure 2B); similar results were obtained by Annexin-V staining and analysis by flow cytometry. Taken together, these results suggest that VEGI signaling is dominant.

VEGI prevents activation of AKT in response to Ang1

AKT has been shown to be an important mediator of Tie2 ligand dependent-[3,12] and independent[14] survival signaling. We next examined whether activation of AKT was altered in response to VEGI. Similar to what others have reported[14], we found that endothelial cells have a basal level of AKT phosphorylation at S473 in normal growth media, which is further augmented following stimulation with Ang1 (Figure 3A, first and second lanes). When we analyzed the attached, arrested population, however, we found that basal phosphorylation of AKT was decreased, while phosphorylation is response to Ang1 remained virtually unaffected (Figure 3A, last fifth and sixth lanes). In the apoptotic, detached population, however, we found that first, total AKT levels were decreased; this is consistent with previous reports that AKT is a substrate for caspases and is degraded during apoptosis[28,29]; the presence of the active p17 caspase 3 subunit confirms that these cells are apoptotic. Secondly, we found that phosphorylation of the remaining AKT is completely inhibited or lost under both basal as well as Ang1-stimulated conditions, even though Tie2 is still detectable. Similar results were obtained using Thr308 p-AKT antibody (data not shown).

We then asked whether restoration of the AKT survival signaling pathway could override VEGI-mediated apoptosis. Transfection of cells with myristoylated Akt was able to confer only about 40% protection from VEGI-mediated apoptosis (Figure 3C). As reported, basal phosphorylation of myristoylated AKT is very high, although we found that it could be further augmented by Ang1 stimulation (Figure 3B). Similar results are found in the VEGI-treated attached population. By contrast, however, we find that basal phosphorylation is

decreased, and Ang1-stimulated prevented, in detached cells undergoing VEGI-mediated apoptosis.

We further determined that treatment with the LY294002 PI3K inhibitor results in loss of full-length Tie2, concomitant with appearance of the 32kDa putative cleavage product, similar to the altered Tie2 profile obtained with VEGI (Figure 3D). Taken together, these data suggest that maintenance of the PI3K pathway is required for continued expression of Tie2, and inhibition of this pathway by VEGI results in Tie2 loss.

Cleavage of Tie2 protein during VEGI-mediated apoptosis

We next wanted to address the mechanism by which VEGI leads to loss of Tie2 expression. We first tested whether loss of Tie2 results from decreased stability. We found that Tie2 protein levels were decreased more rapidly (although less completely) in response to VEGI than following treatment with the protein synthesis inhibitor cycloheximide (Figure 4A), suggesting that loss of Tie2 may result, at least in part, by degradation of Tie2 protein. In addition, as shown in Figure 1B, a c-terminal Tie2 antibody detected an approximately 32kDa band only in the cells undergoing apoptosis in response to VEGI (Figure 4B, arrow) that may represent a c-terminal Tie2 cleavage product. This band appears concomitant to loss of full-length Tie2, but does not appear in untreated cells, cells undergoing growth arrest in response to VEGI, or in cycloheximide-treated cells.

We next sought to address the mechanism by which Tie2 may undergo cleavage during VEGI-mediated apoptosis by testing whether various protease inhibitors could prevent Tie2 loss in response to VEGI. Although both have been previously implicated in apoptotic processes[30,31], neither proteasome inhibitors (lacatacystin and MG132) nor calpain inhibitors

(LL-CHO and ALLN) could prevent the loss of full length Tie2, and the appearance of the putative cleavage product remained unaffected (data not shown). In addition, because it has been reported that Tie1 can undergo metalloprotease (MMP)-mediated cleavage to produce a 46kDa cleavage product that signals independently of ligand[32,33], we tested whether MMPs might be involved in Tie2 loss. We found that the broad-spectrum MMP inhibitor BB-94 could not prevent loss of full length Tie2 or appearance of the 32kDa putative cleavage product in response to VEGI, although it did effectively prevent the appearance of the 46kDa Tie1 cleavage product (data not shown).

It has previously been shown that activation of caspases is required for VEGI-mediated apoptosis, as apoptosis can be attenuated by the broad-spectrum caspase inhibitor ZVAD[26]. We next addressed whether ZVAD could prevent the downregulation of Tie2 by VEGI. As can be seen in Figure 4C (third lane), ZVAD can partially prevent loss of full length Tie2, and completely eliminates the appearance of the 32kDa putative Tie2 cleavage product, suggesting that Tie2 may either act as a direct substrate for caspases, or that loss of Tie2 is downstream of caspase activation. Analysis of the cytoplasmic region of Tie2 shows five potential caspase cleavage sites (Figure 4D): two general DXXD caspase consensus cleavage sites, one I/LETD Caspase 8 consensus site, a newly identified caspase 7 GELE target site[34], and an LXXD motif similar to the LEHD consensus sequence used by caspase 4. In order to identify the caspase(s) that might be involved in Tie2 degradation, we pretreated cells with inhibitory peptides for specific caspases. We found that neither the caspase 3/7 nor the caspase 8 peptide could alter loss of Tie2 or detection of the 32kDa product. By contrast, the group I caspase inhibitor peptides YVAD and LEVD decreased loss of full length Tie2 and significantly reduced the

32kDa product, although the inhibition was not as complete as that observed with ZVAD (Figure 4C).

To further confirm that Tie2 can be cleaved by caspase 4 or other group I-type caspases, recombinant human (rh) Tie2 protein was used as a substrate in an *in vitro* cleavage reaction using recombinant active caspase proteins. Control reactions using recombinant bovine PARP as substrate confirmed the ability of caspases 3, 6, and 7, but not other caspases tested, to generate the canonical 85kDa cleavage product (data not shown). As can be seen in Figure 4E, rh caspase 4, and to a lesser extent rh caspases 1 and 5, were able to cleave rhTie2 *in vitro* (left panel, see arrow). This cleavage occurs in a dose-dependent fashion to give rise to a product that is nearly identical in size to the 32kDa band that is derived from the cleavage of Tie2 in ABAE *in vivo* in response to VEGI (Figure 4E, right panel, arrow), and is prevented by both the ZVAD and LEVD peptide inhibitors. Taken together, these data suggest that part of the mechanism of Tie2 loss during VEGI-mediated apoptosis is cleavage by caspase 4 or a caspase 4-like group I caspase. Interestingly, caspase 7 but not caspase 3 also has the potential to cleave rhTie2 (Figure 4E, asterisk), although the resulting product is much smaller that that generated in response to VEGI and is likely not physiologically relevant to VEGI's effects.

VEGI downregulates Tie2 mRNA

While Tie2 loss occurs in part via caspase-mediated cleavage, the use of the ZVAD and LEVD caspase inhibitors does not completely restore Tie2 levels to that of untreated controls; further, since the cleavage is not observed in response to VEGI-mediated growth arrest but Tie2 levels are decreased, we hypothesized that there was an additional mechanism of Tie2 control. To address this, we analyzed Tie2 mRNA levels in VEGI-treated arrested and

proliferating cells by semi-quantitative RT-PCR. The data show that, similar to the increased Tie2 protein expression with increased confluence, Tie2 mRNA levels are higher in confluent cultures than in proliferating, subconfluent cultures (Figure 5). When confluent cultures are treated with VEGI, the mRNA levels decrease, similar to the decrease in protein expression (Figure 1A). When proliferating cultures are treated with VEGI to induce apoptosis, Tie2 mRNA levels are slightly further decreased; by contrast, induction of apoptosis by 2-methoxyestradiol does not alter Tie2 mRNA levels. These data suggest that decreased Tie2 expression in response to VEGI is controlled, at least in part, by decreased mRNA expression.

DISCUSSION

We have previously demonstrated that elimination of endothelial Tie2 by antisense oligonucleotides results in apoptosis[7], identifying Tie2 as a necessary basal survival factor for endothelial cells. Here we report that VEGI can eliminate Tie2 expression specifically in apoptotic cells, suggesting that VEGI may induce apoptosis, in part, by elimination of Tie2 and its signaling.

We first showed that Tie2 levels are moderately decreased during the growth arrested state induced by VEGI, and that this control of Tie2 expression is likely to be mediated by a decrease in mRNA expression. By contrast, Tie2 levels are nearly eliminated in the apoptotic population by both decrease in mRNA levels as well as caspase-mediated cleavage of Tie2.

We also find that VEGI can prevent the phosphorylation of AKT downstream of Tie2, and that maintenance of this signaling pathway may required for the sustained expression of Tie2.

We surprisingly found that VEGI can prevent signaling from Tie2 even when some Tie2 is present. The mechanism of inhibition of this pathway is yet unclear, although it may be due to 1)inhibition of AKT kinases – We found that AKT phosphorylation was inhibited similarly at both Thr308 and S473, suggesting that level of control lies at PDK1 or above. It is also possible that VEGI is causing activation of AKT phosphatases. Alternatively, the control may be further up in the pathway, such as interference with PI3K association with or activation by Tie2, or activation of the PTEN lipid phosphatase that converts PIP3 to the inactive PIP2 precursor. Future studies will be required to elucidate the upstream mechanism involved in inhibition of Tie2 survival signaling.

The primary level of control seems to be decrease in mRNA levels. Tie2 mRNA is decreased during both growth arrest and apoptosis, although somewhat more fully in the latter case. However, this appears to not be the only means of Tie2 elimination in apoptotic cells; during apoptosis, degradation of Tie2 appears to occur in a caspase-dependent fashion. Since Tie2 levels decrease more rapidly in response to VEGI than following the inhibition of protein synthesis by cycloheximide, Tie2 appears to undergo an active mode of degradation that is prevented by the inhibition of caspase activation by ZVAD. Specifically, this degradation can also be prevented by the inhibition of group I caspases such as caspase 4, but not caspase 3, which has previously been implicated in VEGI-mediated apoptosis[25,26]. Although more commonly associated with the processing of inflammatory mediators[35], caspase 4 and similar group I caspases have also been implicated in apoptotic processes[36,37]. In fact, it has been

shown that activation of caspase 4 precedes, and contributes to the activation of, caspase 3 in response to Fas ligand [38]. The size of the Tie2 product produced *in vitro* by caspase 4 (and weakly generated by caspases 1 and 5) corresponds to the predicted size product (31.2kDa) generated by the use of an LRMD caspase-4-like motif in the cytoplasmic region of Tie2 (Figure 4D) that is conserved between human, bovine, and mouse, but is absent from Tie1 sequence. Further, we have shown that the cytoplasmic region of Tie2 can serve as a substrate for caspases *in vitro*. While there may be a very slight difference in apparent molecular weight of the *in vivo*-cleaved Tie2 and the *in vitro*-generated caspase 4 cleavage product, this may reflect the use of recombinant human Tie2 compared to endogenously-produced bovine Tie2. Accordingly, although the cytoplasmic region of Tie2 contains 19 tyrosines with the potential to be phosphorylated[39], recombinant cytoplasmic Tie2 generated in insects has been reported to be phosphorylated on only two to six of them[40]. A recent report of Tie2 localization to caveolae [41], coupled with reports that caspases are activated in caveolae[42-44], confirm that at least a subpopulation of Tie2 protein may be readily accessible for caspase cleavage following the initiation of apoptosis.

In summary, we have shown that VEGI can effectively prevent Tie2 survival signaling, even in the presence of Ang1. To our knowledge, VEGI is the first endogenous negative regulator of Tie2 thus far identified. Mechanistically this control occurs via inhibition of AKT signaling, which in turn leads to downregulation of Tie2 both by decrease in mRNA as well as caspase-dependent degradation of Tie2. It is expected that VEGI may therefore be effective as an anti-angiogenic agent against Tie2-dependent tumors. These data, coupled with our previous reports that VEGI can prevent bFGF and VEGF-stimulated angiogenesis in vivo[24],

and prevent tumor growth in xenograft[23,24] and preclinical models (in preparation), suggest that VEGI may have use as a broad-spectrum anti-angiogenic therapeutic agent for the treatment of cancer.

Figure 1. Tie2 levels are moderately decreased during VEGI growth arrest, but nearly eliminated during VEGI-induced apoptosis. A, Density-arrested ABAE were replated at low density and treated with VEGI (90ng/ml) at various times after release. At the indicated times, the detached and adherent cells were harvested collected together and lysates subjected to Western analysis for Tie2. Left panels, VEGI was added immediately following replating ('Released from Density Arrest'). Middle panels, Cells were treated 96 hours after replating, when cells were in log phase growth ('Proliferating'). Right panels, Cells were allowed to once again become confluent and density arrested (6 or 7 days after replating, 'Confluent'), after which VEGI was added. B, Proliferating ABAE cells were treated with VEGI (90ng/ml) or control buffer for 12 hours. Either both floating and adherent cells were harvested together as above ('All'), or detached apoptotic cells ('Detached') were harvested separately from the arresting, adherent cells ('Adherent') and lysates analyzed by Western for Tie2 using both c-terminal and n-terminal antibodies. Western for p17 active subunit of caspase 3 designates apoptotic cells, and β Actin serves as loading control. The experiments were repeated at least two times.

Figure 2. Angl cannot reverse the effect of VEGI. A, Proliferating ABAE were replated into normal growth media, pretreated or not 1 hour with indicated concentration of Angl ('A'), then treated with VEGI ('V') to induce apoptosis. Lysates were harvested after 12 hours and subjected to Western. Results are representative of three experiments. B, Proliferating ABAE were plated into normal growth media in 96 well plates and pretreated or not with Angl for 1 hour, after which VEGI (maroon bars) or control buffer (blue bars) was added to the media. After 16h, caspase 3/7 activity was measured by adding Z-DEVD-Rhodamine110 peptide in a

lysis buffer directly to the media. Fluorescence (EM 485nm, EX 550nm) was measured on a Wallac 1420 plate reader. Results are mean of triplicate wells from two experiments +/- SD.

Figure 3. VEGI prevents activation of AKT in apoptotic cells. A, Proliferating ABAE cells were treated with VEGI (90ng/ml) or control buffer for 6 hours, then stimulated with Ang1(200ng/ml) or PBS for 20 minutes. Detached apoptotic cells ('Detached') were harvested separately from the arresting, adherent cells ('Adherent'). Lysates were analyzed by Western for phosphorylated AKT, total AKT and the active p17 subunit of caspase 3; β actin serves as loading control. B, ABAE were transiently transfected with both pCMV-SPORT-6-Tie2 and pcDNA3.1-myr-AKT, or pcDNA3.1 alone using Effectene. Twenty-four hours after transfection, cells were treated with VEGI (90ng/ml). After 8 hours of treatment, cells were stimulated or not with Ang1 (200ng/ml) for 20 minutes, after which cells were harvested, collecting adherent control buffer-treated cells, and separately harvesting VEGI-treated detached cells ('Detached') or VEGI-treated adherent cells ('Adherent') as above. Western analysis for p-AKT and AKT was performed. The experiments were repeated at least twice. C, ABAE transfected with myristoylated Akt were replated in the presence of VEGI (90ng/ml) and caspase 3/7 activity was measured by adding Z-DEVD-Rhodamine110 peptide in a lysis buffer directly to the media. Fluorescence (EM 485nm, EX 550nm) was measured on a Wallac 1420 plate reader. D, ABAE were treated with the indicated concentration of LY294002 ('LY') for 12 hours, then lysates isolated and subjected to Western analysis for Tie2.

Figure 4. Tie2 is Degraded during VEGI-mediated apoptosis. A, Ninety-six hours following release from density arrest, proliferating ABAE cells were treated with VEGI to

induce apoptosis; identical cultures were treated with cycloheximide (CHX, 3μg/ml). Cells were harvested 6 or 24 hours following addition of agent and lysates were subjected to Western analysis for Tie2. B, Similar experiment to 'A', showing full length p140 Tie2 and the detection of a putative Tie2 cleavage product (arrow). C. Broad-spectrum ZVAD or caspase 4 inhbitor LEVD decreases loss of Tie2 and nearly eliminates appearance of the cleavage product. Proliferating ABAE were treated with the indicated caspase inhibitor, then treated with VEGI (90ng/ml) for 16 hours. Floating and adherent cells were collected together and lysates subjected to Western analysis for Tie2. Full-length p140kDa Tie2; arrowhead indicates putative Tie2 cleavage product. The experiment was repeated twice. D, Amino acid sequence of the Tie2 cytoplasmic region. Potential caspase cleavage sites are bolded. E, Tie2 can serve as a caspase 4 substrate in vitro. In vitro cleavage reaction using recombinant caspases and recombinant cytoplasmic regions of Tie2 ('p42 rhTie2') or PARP as substrate were performed as described in 'Materials and Methods' and subjected to Western analysis as indicated; arrow designates cleavage product. First lane, right panel shows lysate from Tie2 VEGI-treated ABAE cells for comparison of cleavage product size. Results are representative of three experiments.

Figure 5. Tie2 mRNA levels are decreased by VEGI. Profliferating ('Prolif') or confluent ('Arrested') ABAE were treated with VEGI (100ng/ml) or 2-methoxyestradiol (2-ME) for 6 hours, after which RNAs were isolated and subjected to semi-quantititative RT-PCR for Tie2. GAPDH serves as control. Experiment was performed twice. Similar results were obtained using a different set of Tie2 primers.

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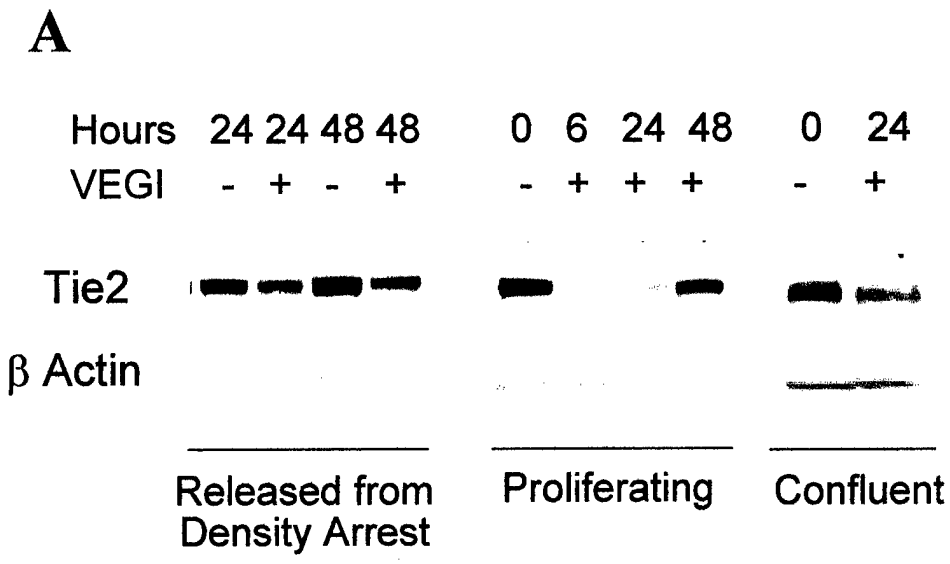
Figure 1


A
Hours 24 24 48 48 0 6 24 48 0 24
VEGI - + - + - + + + - +
Tie2
β Actin
Released from Density Arrest
Proliferating
Confluent

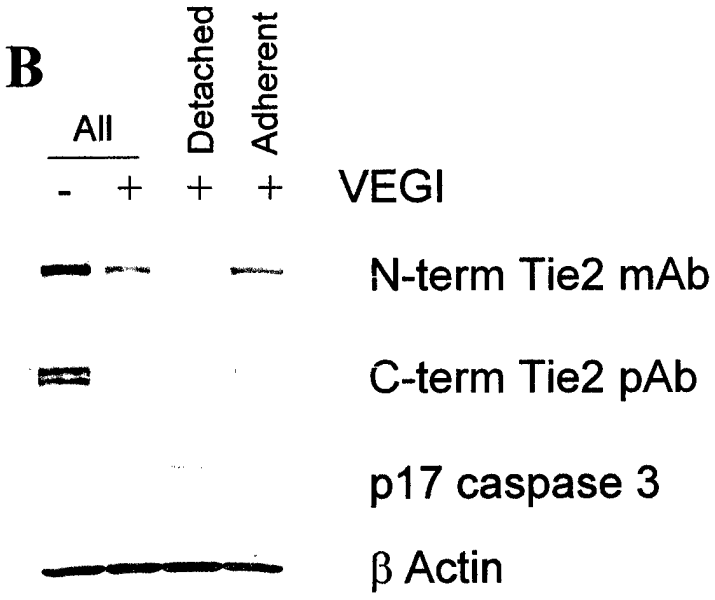
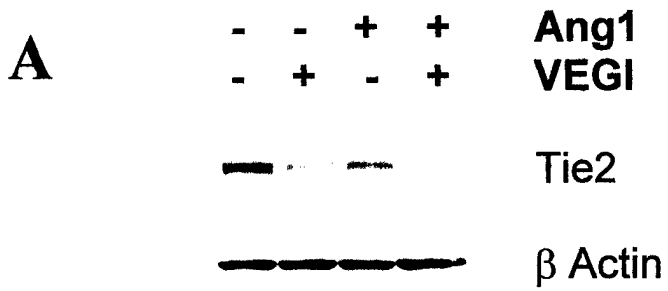

B
All
Detached
Adherent
- + + + VEGI
N-term Tie2 mAb
C-term Tie2 pAb
p17 caspase 3
β Actin

Figure 2


A
- - + + Ang1
- + - + VEGI
Tie2
β Actin

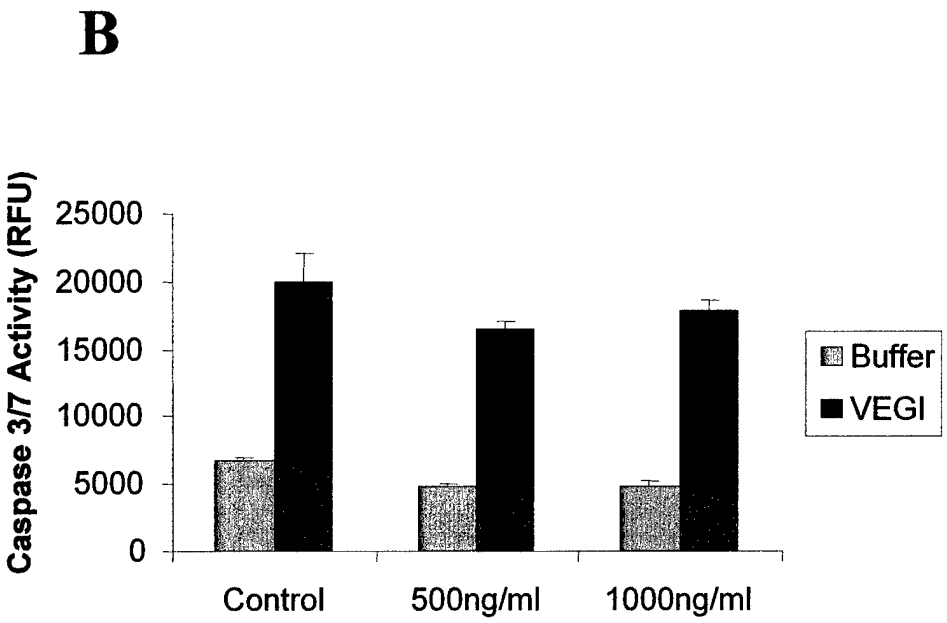

B
Caspase 3/7 Activity (RFU)
25000
20000
15000
10000
5000
0
Control
500ng/ml
1000ng/ml
Buffer
VEGI

Figure 3

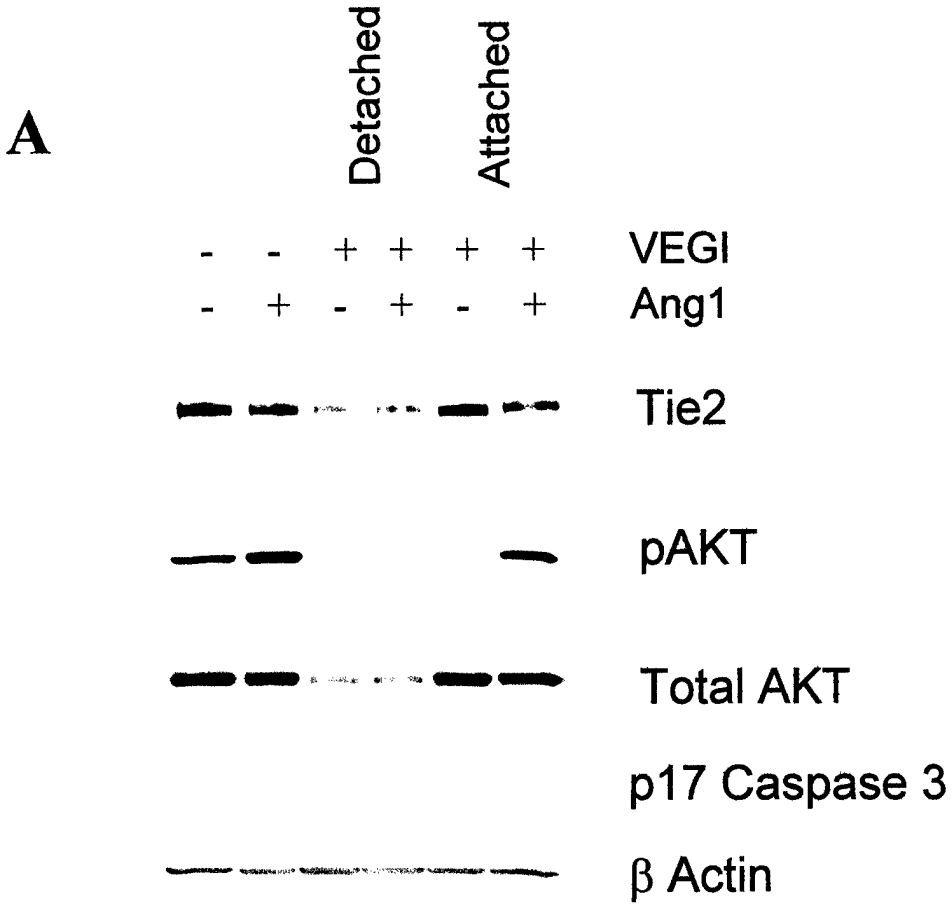

A
Detached
Attached
- - + + + + VEGI
- + - + - + Ang1
Tie2
pAKT
Total AKT
p17 Caspase 3
β Actin
B
Detached
Attached
- - + + + + + + Tie2/myrAKT
- - - - + + + + VEGI
- + - + - + - + Ang1
p-AKT
Total AKT

Figure 3

C

D

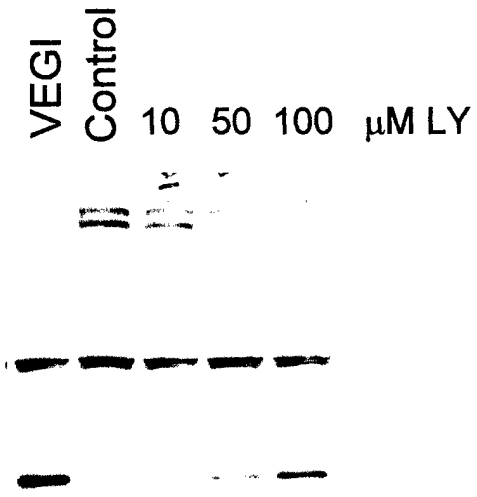
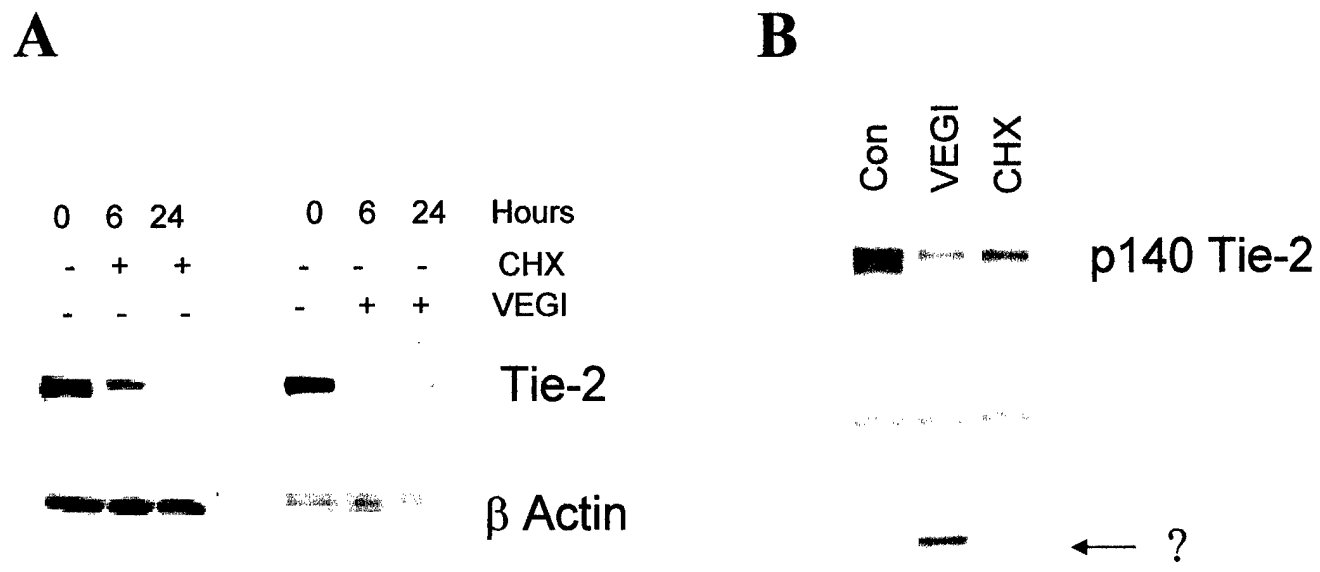


Figure 4


A
0 6 24
0 6 24 Hours
- + + - - - CHX
- - - - + + VEGI
Tie-2
β Actin
B
Con
VEGI
CHX
p140 Tie-2
?

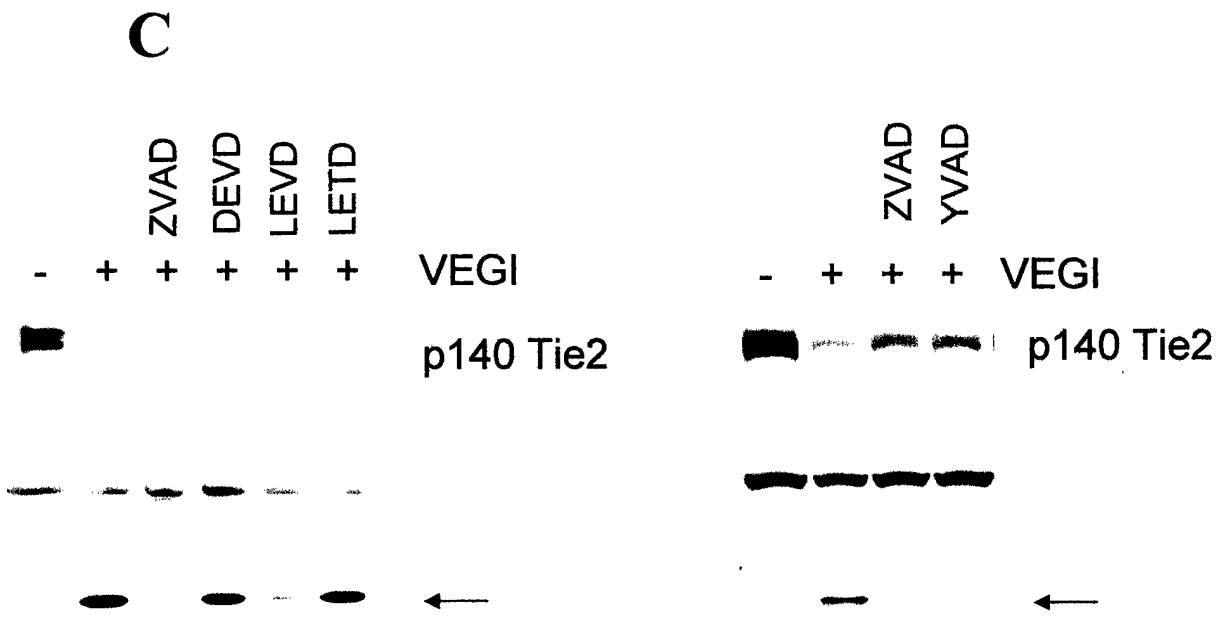

C
ZVAD
DEVD
LEVD
LETD
- + + + + + VEGI
p140 Tie2
ZVAD
YVAD
- + + + VEGI
p140 Tie2

Figure 4

D

721 ssnptsshel ttlsesqapa dlggrkmlli ailgsagmtc ltvllaflim lqlkranvqr

781 rmaqafqnvr eepavqfnsg tlalnrkakn npdptiypvl **dwnd**ikfqdv igegnfgqvl

841 karikkdg**LR MD**aaikrmke yaskd**dhrd**f a**gele**vlckl ghhpniinll gacehrgyly

901 laieyaphgn lldflrksrv **letd**pafaia nstastlssq qllhfaadva rgmdylsqkq

961 fihrdlaarn ilvgenyvak iadfglsrgq evyvkktmgr lpvrwmaies lnysvyttns

1021 dvwsygvllw eivslggtpy cgmtcaelye klpqgyrlek plncddevyd lmrqcwrekp

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E

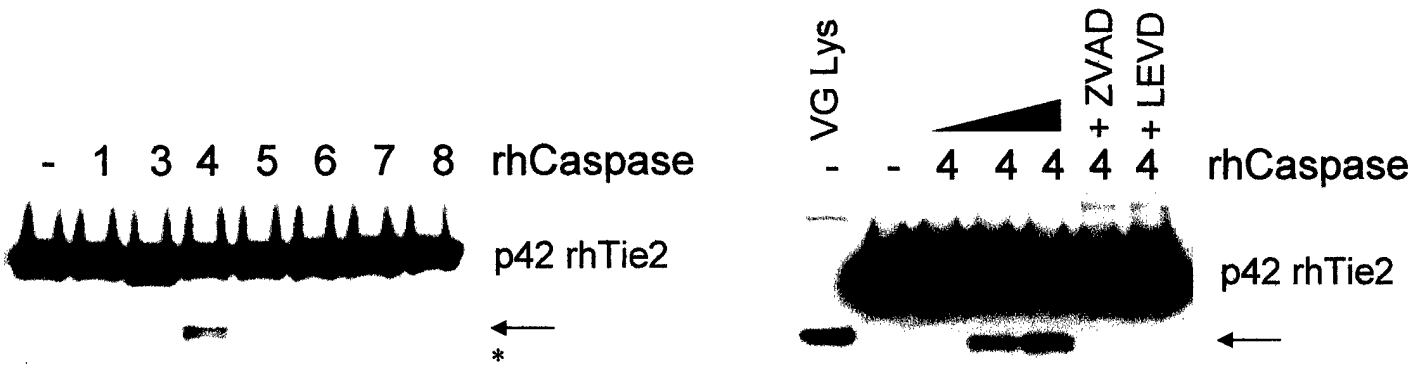
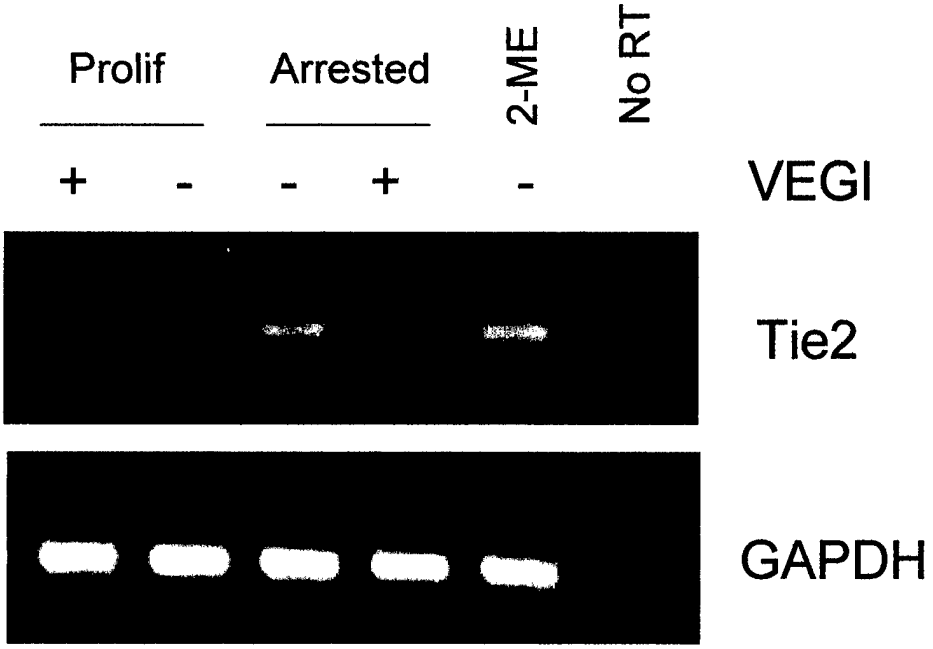


Figure 5



1: Cell Res. 2003 Oct;13(5):309-17.

Related Articles, Lir

Cell Research (full-text)

The enigmatic role of angiopoietin-1 in tumor angiogenesis.

Metheny-Barlow LJ, Li LY.

Department of Oncology, Georgetown University Medical Center, Washington, DC 20007, USA.

A tumor vasculature is highly unstable and immature, characterized by a higl proliferation rate of endothelial cells, hyper-permeability, and chaotic blood flow. The dysfunctional vasculature gives rise to continual plasma leakage ar hypoxia in the tumor, resulting in constant on-sets of inflammation and angiogenesis. Tumors are thus likened to wounds that will not heal. The lack of functional mural cells, including pericytes and vascular smooth muscle cells, in tumor vascular structure contributes significantly to the abnormality of tumor vessels. Angiopoietin-1 (Ang1) is a physiological angiogenesis promoter during embryonic development. The function of Ang1 is essential t endothelial cell survival, vascular branching, and pericyte recruitment. However, an increasing amount of experimental data suggest that Ang1-stimulated association of mural cells with endothelial cells lead to stabilizatic of newly formed blood vessels. This in turn may limit the otherwise continuous angiogenesis in the tumor, and consequently give rise to inhibitio of tumor growth. We discuss the enigmatic role of Ang1 in tumor angiogenesis in this review.

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